

Variations in Copepod Proteome and Respiration Rate in Association with Diel Vertical Migration and Circadian Cycle

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Abstract. The diel vertical migration of zooplankton is a process during which individuals spend the night in surface waters and retreat to depth during the daytime, with substantial implications for carbon transport and the ecology of mid-water ecosystems. The physiological consequences of this daily pattern have, however, been poorly studied beyond investigations of speed and the energetic cost of swimming. Many other processes are likely influenced, such as fuel use, energetic trade-offs, underlying diel (circadian) rhythms, and antioxidant responses. Using a new reference transcriptome, proteomic analyses were applied to compare the physiological state of a migratory copepod, *Pleuromamma xiphias*, immediately after arriving to the surface at night and six hours later. Oxygen consumption was monitored semi-continuously to explore underlying cyclical patterns in metabolic rate under dark-dark conditions. The proteomic analysis suggests a distinct shift in physiology that reflects migratory exertion and changes in metabolism. These proteomic analyses are supported by the

respiration experiments, which show an underlying cycle in metabolic rate, with a peak at dawn. This project generates molecular tools (transcriptome and proteome) that will allow for more detailed understanding of the underlying physiological processes that influence and are influenced by diel vertical migration. Further, these studies suggest that *P. xiphias* is a tractable model for continuing investigations of circadian and diel vertical migration influences on plankton physiology. Previous studies did not account for this cyclic pattern of respiration and may therefore have unrepresented respiratory carbon fluxes from copepods by about 24%.

Introduction

One of the greatest cyclical patterns in the pelagic ecosystem is the daily migration of various zooplankton and fish to depth, a process referred to as diel vertical migration (DVM). DVM is typified by the presence of transient species in the warm, oxygenated, photic zone during the night, followed by a movement of individuals into colder, deeper water during the day (although variations, including reverse migrations, exist; Heywood, 1996; Hays, 2003). These daily migrations are thought to be energetically expensive, with individuals smaller than 1 cm traveling hundreds of meters per day. DVM speed has been estimated, *via* calculations of the observed amplitude and duration of DVM for copepods and euphausiids, to range between 10 and 55 mm s⁻¹ (Wiebe *et al.*, 1992; Mauchline, 1998). The estimates of the physiological costs of such profound exertion, based on force transducer and video analyses, range between 13% and 120% of copepod basal metabolic rate (reviewed in Mauchline, 1998). Despite the costs, the process is believed to provide a number of advantages to migratory or-

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Abbreviations: ACN, acetonitrile; ANOSIM, analysis of similarity; BATS, Bermuda Atlantic Time Series; BUSCO, Benchmarking Universal Single-Copy Orthologs; DM, dry mass; DVM, diel vertical migration; FFT-NLLS, fast Fourier transform non-linear least squares; GO, gene ontology; MESA, maximum entropy spectral analysis; NAD, nicotinamide adenine dinucleotide; NAD⁺, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NMDS, non-metric multidimensional scaling; nr, non-redundant database; NSAF, normalized spectral abundance factor; RT, room temperature; TTP, Trans Proteomic Pipeline.

Online enhancements: data files.

ganisms, including niche partitioning, metabolic advantage due to colder temperatures at depth, retreat from light or high temperatures, and, most importantly, predator avoidance (Hays, 2003; Antezana, 2009).

Globally, the DVM of the zooplankton community is estimated to be responsible for 15%–40% of the total organic carbon export from the surface to the mesopelagic (Steinberg *et al.*, 2000; Bianchi and Mislan, 2016), exceeding in many cases the carbon flux related to passively sinking particles (Kobari *et al.*, 2003, 2008; Steinberg *et al.*, 2008; Steinberg and Landry, 2017). Similar percentages are reported for the nitrogen-related processes (*e.g.*, Dam *et al.*, 1995; Al-Mutairi and Landry, 2001; Schnetzer and Steinberg, 2002b; Steinberg *et al.*, 2002). These estimates are typically made by applying mass-specific and temperature-scaling factors to experimentally calculated oxygen consumption, as well as nitrogen and carbon excretion by individual organisms. These experiments are traditionally done by placing individuals in bottles for long durations (8–24 h), with no mention of the start time of the experiment; and, thus, variations in physiological state that are a consequence of circadian patterns or the DVM process are lost. If there are substantial physiological changes associated with migration or hidden daily cycles in major physiological processes, such as oxygen consumption and ammonia excretion, they may cause errors in the estimations of organismal contributions to biogeochemical flux during daytime at depth. Previous studies have suggested that this may indeed be the case, with the migratory copepods *Pleuromamma xiphius* and *Calanus euxinus* showing substantially higher respiration rates late in the evening (22:00) and early in the morning (05:00) relative to the nadir at mid-day (13:00) (Pavlova, 1994). The design of these studies still relied upon noncontinuous respirometry measurements (3–6-h endpoint incubations), making a pinpointed analysis of the variation in physiological response impossible.

To explore the physiological signature of DVM, more finely resolved measurements are required. Furthermore, although it is important to define the energetic cost of swimming, understanding the full physiological implications of migrations requires analysis of more complex mechanisms such as changes in metabolic pathways, endogenous circadian rhythms, and antioxidant activity. These topics have been explored in terrestrial migratory studies (*i.e.*, Gwinner and Helm, 2003; McGuire and Guglielmo, 2009; Skrip and McWilliams, 2016) but have not, to date, received much attention in zooplankton because of the difficulty of organismal sampling, the small size of individuals, and the lack of appropriate molecular tools to adequately quantify changes. Under natural conditions, timing of planktonic DVM is largely cued by daily light-dark cycles, and over time animals become synchronized, or entrained, to this predictable environmental cue. By showing that daily movement patterns can persist in migratory crustaceans in constant darkness, laboratory-based studies have indicated that DVM could be regulated by an endogenous

circadian clock (Enright and Hamner, 1967), thus suggesting that there is still much to be learned in this system.

New advances in molecular techniques have recently opened the door for more detailed analyses of these more complex aspects of DVM, and research regarding the influence of circadian patterns on diel and seasonal migrations is underway (Cohen and Forward, 2009; Teschke *et al.*, 2011; Christie *et al.*, 2013; De Pitta *et al.*, 2013). A recent analysis using semi-continuous optical oxygen sensing, characterization of DVM behavior, and expression of core clock genes *via* quantitative polymerase chain reaction (qPCR) revealed that the copepod *Calanus finmarchicus* has a true circadian rhythm in metabolism, with a suggested peak in respiration during the early evening (Häfker *et al.*, 2017). This sets the stage for studies that investigate which other processes may also be influenced by both migratory behavior and circadian cycles.

The goal of this project was to determine whether it is possible to observe biogeochemically relevant diel changes in the physiology of a subtropical copepod through organismal (respiration) and molecular (proteomics) methods. To support the latter analysis, we generated a species-specific copepod transcriptome that could then be used to identify copepod proteins and to detail the change in protein abundances that a vertical migratory zooplankton experiences over the nighttime portion of its diel cycle. *Pleuromamma xiphius*, a well-known vertical migrator at the Bermuda Atlantic Time Series (BATS) site, was chosen for this study because it has previously been shown to have diel patterns in respiration rate that are assumed to be associated with its migratory behavior (Pavlova, 1994). This species is abundant in the Sargasso Sea, and its contributions to nitrogen and carbon flux have previously been characterized using classical bottle sampling methods (Wormuth, 1981; Steinberg *et al.*, 2000, 2002; Schnetzer and Steinberg, 2002b; Teuber *et al.*, 2013), providing greater context for the more detailed physiological assessment enabled by proteomics analysis.

Materials and Methods

Copepod collection for transcriptomic and proteomic analyses

Female copepods (*Pleuromamma xiphius* (Giesbrecht, 1889)) were collected off the coast of Bermuda (31°39.93N, 64°00.14W; 31°32.00N, 63°37.02W) near the BATS site. Sampling was limited to the nighttime because the available net systems precluded capture of healthy organisms from their daytime depth, which is typically ~400–650 m locally (LB-B and AM, unpubl. data). On April 17, 2016, a 1-m² square net with a Star-Oddi depth logger (Gardabaer, Iceland) was towed horizontally at 02:45 (local time) to a 120-m depth, then retrieved, with two 6-min pauses to try to target regions below the chlorophyll *a* maximum (as observed *via* nearby hydrographic sampling). On April 18 at 20:30 (local time), after a

~19:45 sunset, the same net was deployed to 180 m, towed, and retrieved in an identical fashion. Upon recovery of the net, the cod end was diluted in a 20-L cooler containing water from the flow-through seawater line. Individuals were then manually captured *via* a soft pipette and identified under a Leica stereomicroscope (Wetzlar, Germany) to verify species identity and exclude individuals with broken antennae or legs. The copepods were immediately individually flash-frozen in a dry shipper filled with liquid nitrogen. Sorting of all individuals used in subsequent analyses was completed 32 and 34 min after capture (on April 17 and 18, respectively). For simplicity, these samples are subsequently referred to as 02:00 and 20:00.

Respiration experiment

An additional collection was made offshore from Bermuda (32°20.28N, 64°32.82W) from a small vessel on May 11, 2017, between 21:00 and 23:00. Sunrise was 06:23, and sunset was 20:07. Three tows were conducted with a specialized 150- μm mesh Reeve net (Sea-Gear, Melbourne, FL) with a large cod end. The net was slowly towed horizontally at a 100-m depth for 15 min and then retrieved. Animals were gently poured through a 1000- μm mesh sieve and transferred to a cooler containing *in situ* water. After the third tow, organisms were brought back to the laboratory, and *P. xiphias* were picked out of the coolers. Copepods were placed at low densities (<10 individuals L^{-1}) in 0.2- μm filtered *in situ* water and were maintained in a dark incubator at 20 °C (temperature at the chlorophyll *a* maximum) for 1 h to allow for gut clearance and acclimatization (Bautista and Harris, 1992; Irigoien, 1998). After the acclimatization period, individuals were transferred to fresh 20 °C 0.2- μm filtered *in situ* water in 60-mL glass Biological Oxygen Demand (BOD) bottles (Wheaton, Millville, NJ) containing an optically sensitive oxygen sensor (OXFOIL; PyroScience, Aachen, Germany). As a control for bacterial respiration, every fourth chamber was filled with water but was left without a copepod. Chambers were placed in a dark 20 °C incubator, and the oxygen concentration in each chamber was then measured noninvasively and continuously (every 2 min) for 3 d by using a FireSting optical oxygen meter (PyroScience, Aachen, Germany). At the end of the experiment, individuals were visually inspected to ensure that they were swimming. The respiration rate for each individual ($n = 4$) was corrected for bacterial respiration (on average, 0.06 $\mu\text{mol h}^{-1}$) by subtracting the oxygen concentration within the organismal chamber from the control chamber at each time point. Periodicity was visualized by plotting the oxygen consumption rate ($\mu\text{mol O}_2 \text{h}^{-1}$) calculated over a 2-h period. These rates were then binned by 1 h and plotted *versus* the midpoint of the calculation period.

To illustrate how the duration or starting point of the respiration experiment may influence calculation of zooplankton respiratory active flux, typical endpoint calculations were made ($\text{Oxygen}_{\text{initial}} - \text{Oxygen}_{\text{final}} / \text{Duration of experiment}$). Starting values ($\text{Oxygen}_{\text{initial}}$) were chosen as the first measurement

(taken at 02:30), then, starting at 06:00 on the first day, every 6 h for 2 days. Ending values ($\text{Oxygen}_{\text{final}}$) were taken 6, 12, 18, or 24 h after the starting time point, except in the case of the 02:30 measurement, which was calculated using endpoints 3.5, 9.5, 15.5, and 21.5 h after the start of the experiment. Oxygen content during these time periods declined from 100% to a minimum of 70%. The use of later time points was excluded in case there was an influence of reduced oxygen availability in the chambers on metabolic rate. This is well above the critical oxygen partial pressure (p_{crit}) typical of most marine invertebrates (Childress and Seibel, 1998) and well above the p_{crit} measured for the congener *Pleuromamma abdominalis* at 23 °C (<30% oxygen saturation; Kiko *et al.*, 2016).

Rhythmicity in respiration was evaluated through period analysis as implemented on the BioDare2 web portal (BioDare2, 2016). Linear de-trending was conducted to compensate for the decline in respiration rate over time, which might otherwise obscure detection of rhythmicity (Zielinski *et al.*, 2014). De-trended time series were evaluated using the fast Fourier transform non-linear least squares (FFT-NLLS) algorithm and maximum entropy spectral analysis (MESA) methods. These methods were chosen because they provide error estimates for the period, because they are based on different principles (curve fitting *vs.* stochastic modeling), and because MESA is relatively robust to the presence of baseline trends (Zielinski *et al.*, 2014). Statistical significance of a 24-h period was tested using a waveform-independent nonparametric approach within the R package RAIN, with the “longitudinal” analysis option (Thaben and Westermark, 2014).

Transcriptome generation

Two males and one female from both the 02:00 and 20:00 capture time points were extracted in two mixed batches of three individuals using the Qiagen universal RNA mini kit (Qiagen, Germantown, MD). A 5- μL aliquot of each extraction was combined (for a total of 6 individuals) and sent to the University of Rochester Genomics Research Center, where a library was constructed using the TruSeq library prep kit (Illumina, San Diego, CA). The cDNA library was sequenced on one lane on an Illumina HiSeq2000 with 125-bp paired-end reads. Raw sequences were quality filtered by the sequencing facility using the program Trimmomatic (ver. 0.32; Bolger *et al.*, 2014). The program was set to eliminate adapter sequences (2:30:10) and to discard low-quality scores using a sliding window (4:20) that removed both trailing and leading sequences (13) and left only sequences with a minimum length of 15 for downstream use. These cleaned sequences were used to assemble a transcriptome using the Trinity software package (ver. 2.1.1; Grabherr *et al.*, 2011) with default parameters (k-mer = 25; cutoff = 200 bp). The transcripts within this assembly were clustered with the program CD-HIT EST (ver. 4.6.1; Li and Godzik, 2006) to a similarity of 95%. The

quality of the transcriptome, read depth, and estimates of expressed genes were explored using the downstream scripts packaged with Trinity and with Benchmarking Universal Single-Copy Orthologs (BUSCO), using default parameters and the metazoan lineage file (ver. 3.0.2; Simão *et al.*, 2015; Waterhouse *et al.*, 2018).

Protein digestion, desalting, and liquid chromatography-tandem mass spectrometry

To reduce variability within time points, analysis was restricted to adult female copepods ($n = 6$ per time point). These were transported on dry ice to the University of Washington for proteomic analysis. Each copepod was homogenized in 50 mmol L⁻¹ NH₄HCO₃ (100 mL) before 6 mol L⁻¹ urea was added. Sonication, digestion, and desalting proceeded as previously described (Timmins-Schiffman *et al.*, 2013), with the additional digestion enzyme LysC (Wako Chemicals, Richmond, VA). Briefly, after sonication and protein quantification, tris-HCl (1.5 mol L⁻¹, pH 8.8) and tris (2-carboxyethyl)phosphine were added to each sample and incubated for 1 h at 37 °C. Iodoacetamide was added to alkylate the proteins and incubated for 1 h in the dark at room temperature (RT). Excess iodoacetamide was absorbed over a 1-h incubation at RT by dithiothreitol. To reduce the number of undigested cleavage sites, samples were preliminarily digested by LysC for 1 h at RT at a 1 : 20 enzyme : protein ratio. After LysC digestion, NH₄HCO₃ and high-performance liquid chromatography-grade methanol were added to each sample to allow trypsin digestion. Trypsin was added in a 1 : 20 ratio and incubated overnight at RT. The next day, samples were evaporated, reconstituted in 5% acetonitrile (ACN) with 0.1% trifluoroacetic acid, and desalted using a MacroSpin Column (Nest Group, Southborough, MA), following the manufacturer's instructions. Desalted peptides were dried and reconstituted in 100 μL of 5% ACN with 0.1% formic acid.

The mass spectrometry analysis was performed at the University of Washington Proteomics Resource (Seattle, WA). All 12 samples were analyzed on a Q Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA) under data-dependent acquisition mode, injecting equal quantities of peptide (1 μg) in triplicate. Peptides were eluted using an acetonitrile gradient (5%–85%) over the course of 75 min.

Transcriptome and proteome annotation

The transcriptome assembly was annotated using a BLASTP search against a local UniProt TrEMBL database (UniProt Consortium, 2016), with an e-value cutoff of 1e⁻¹⁰ (Camacho *et al.*, 2009). It was further annotated using a local BLASTX *versus* the National Center for Biotechnology Information (NCBI; Bethesda, MD) non-redundant (nr) database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/; downloaded from NCBI on January 19, 2017), with an e-value cutoff of 1e⁻⁵. To provide

more comprehensive cross annotation with closely related pelagic rustaceans, the assembly was reciprocally blasted using TBLASTX (NCBI) and an e-value cutoff of 1e⁻⁵ *versus* *Calanus finmarchicus* (GenBank accession no. GBFB01000001.1; Tarrant *et al.*, 2014), *Euphausia superba* (available from the European Bioinformatics Institute as study PRJEB6147, HACF01000001–HACF01058581; Meyer *et al.*, 2015), and *Meganyctiphanes norvegica* (GenBank accession no. GETT00000000.1; Blanco-Bercial and Maas, 2018) transcriptomes. We also performed a BLASTX search with an e-value cutoff of 1e⁻⁵ against protein sequences from the well-annotated *Drosophila melanogaster* using Flybase (<http://flybase.org>, FB2016_03). Gene families of specific interest (*i.e.*, circadian, molting, metabolic, stress response) in pelagic crustaceans have previously been identified from transcriptomic resources by Meyer *et al.* (2015) and Christie *et al.* (2013). These are gene families that are likely to be influenced by diel changes in physiology; therefore, homologs of these genes were identified using the previously described BLASTX and TBLASTX searches of the *E. superba* assembly or Flybase, respectively.

Peptide identifications and protein inferences for tandem mass spectra for each copepod were found through Comet (2016.01 rev. 2; Eng *et al.*, 2013, 2015) searches and the Trans Proteomic Pipeline (TPP) (Deutsch *et al.*, 2010) against *P. xiphias* predicted protein sequences, translated from the assembled transcriptome. Comet parameters included a concatenated decoy search against fully digested tryptic peptides, allowing for two missed cleavages (for full Comet parameters see Supplemental Data 1; Supplemental Data files 1–4 are available online). A probability cutoff at the peptide level of 0.9 was implemented in the TPP. Abacus was used to coordinate protein inferences across replicates and calculate the normalized spectral abundance factor (NSAF) (Fermin *et al.*, 2011) for proteins with a minimum combined probability of at least 0.9. Only proteins with at least two spectral counts across all replicates were included in further analysis. The *P. xiphias* proteome (based on inferred proteins detected through mass spectrometry) was independently annotated with BLASTP against the UniProt TrEMBL database (UniProt Consortium, 2016) with an e-value cutoff of 1e⁻¹⁰. Annotation-associated gene ontology (GO) (du Plessis *et al.*, 2011) annotations were downloaded from UniProt (UniProt Consortium, 2016).

Protein abundance analysis

Individual copepod protein abundance profiles were compared using non-metric multidimensional scaling (NMDS) and analysis of similarity (ANOSIM) by using the vegan library in R, version 3.3.2 (Oksanen *et al.*, 2016; R Core Team, 2016). NSAF values were log($x + 1$) transformed, and a NMDS plot was generated using a Bray-Curtis dissimilarity matrix. Differential abundance of proteins was determined using QSpec (Choi *et al.*, 2008). Spectral counts were summed across technical replicates within biological replicates (cope-

pods) in the QSpec analysis. A protein was considered differentially abundant if its log-fold change was $>|0.5|$ and its z -value was $>|2|$.

Functional enrichment analysis was completed for GO terms associated with differentially abundant proteins. Two protein sets (positive and negative log-fold change) were compared to detect proteins to determine protein functional groups that were overrepresented in the differentially abundant proteins compared to the background. The analysis was performed using an enrichment tool developed in-house and described in Timmins-Schiffman *et al.* (2017). Briefly, a P -value was calculated describing the enrichment of a GO term in a set of tested proteins above what would be expected by chance, given the annotation of the *P. xiphias* proteome. A P -value cutoff of 0.01 was used to ascribe statistical significance to GO terms representing biological process, cellular component, or molecular function.

Results

Respiration experiment

Continuous monitoring of *Pleuromamma xiphias* oxygen consumption from 4 separate individuals for 3.5 d under full dark conditions revealed a consistent ~ 24 -h oscillation in respiration rate, although the circadian signal was dampened and disappeared over time (Fig. 1A). This may reflect attenuation of the cycle in the absence of an external light cue, artifacts associated with continued monitoring in a small enclosure, or some combination of factors. There was a failure in one of the oxygen meters on the first day (14:39–15:17), leaving a small gap in the measurement of three individuals; however, a cyclical pattern of metabolic rate, with a peak near dawn (local sunrise was 06:23) and a nadir at mid-evening was apparent for all four copepods. Oxygen content in the chambers

had fallen from 100% to $\sim 70\%$ saturation by the beginning of the third day. All individuals were swimming and responsive to touch at the end of the experiment.

Following linear de-trending, 2 complementary methods identified near 24-h periodicity in respiration rate (FFT-NLLS: 25.98 ± 3.83 SD; MESA: 26.48 ± 4.84 SD; FFT-NLLS curve fit shown in Fig. 1B). Nonparametric testing of the mean respiration rate indicated a highly significant 24-h period ($P = 1.74 e^{-15}$). Individual profiles also showed significant 24-h periodicity, and the result was robust in the presence of the overall declining respiration rate.

In order to evaluate the impact of circadian patterns on analyses of respiration rate, comparisons were made among metabolic rates calculated using varying starting times for the first 2.5 d of the experiment. These analyses reveal diel changes in rate as well as a decrease in oxygen consumption over time that was likely a consequence of typical starvation and captivity stress (Fig. 2A). The discrepancies between values that were calculated over a 24-h interval but with different starting times (which should be the same because they integrate over the full circadian cycle) were used to back-calculate the change in rate due to the captivity effect as

$$y = 3e^{-11}x^{5.3367},$$

where x = duration of captivity and y = decline in metabolism attributable to captivity ($R^2 = 0.955$). If the metabolic rate calculations are corrected by this simplified approximation of the captivity effect (Fig. 2B), it is obvious that the starting time of the respiration experiment has an important impact on the calculation of metabolic rate in the first 6 h (50% higher if the experiment started in the early morning). If the calculation is extended to 12 h, this effect is substantially reduced (ranging from 24% higher if it was started in early morning to 6% lower if it was started in late afternoon).

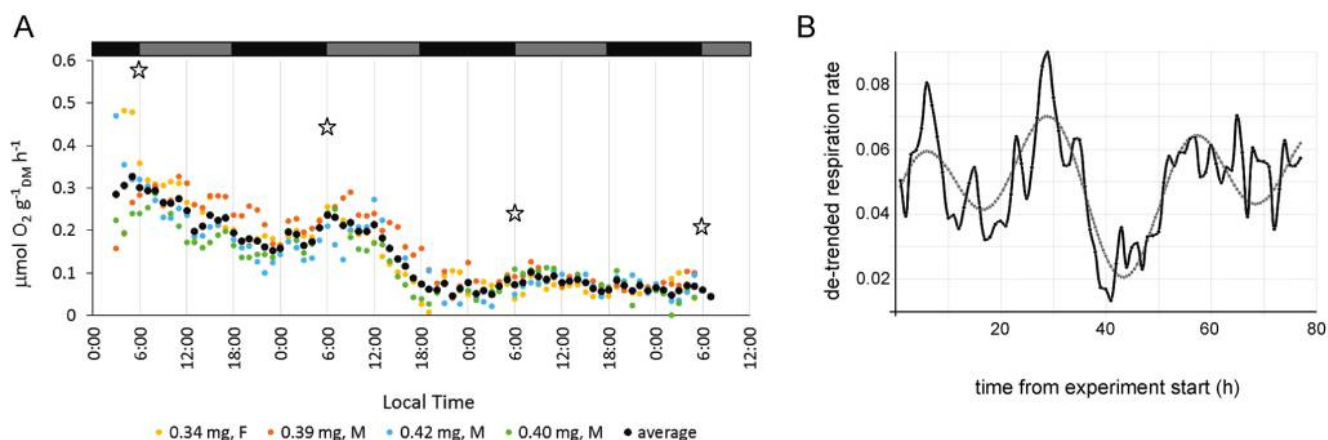


Figure 1. Circadian pattern in respiration rate. (A) Mass-specific respiration rate of 4 individuals maintained for 3 days in darkness at 20 °C. The dry mass and sex of each individual are noted in the legend. Periodicity was visualized by plotting 1-hour binned averages of the respiration rate ($\mu\text{mol O}_2 \text{ g}^{-1} \text{ DM h}^{-1}$). (B) Curve fit (gray line) to de-trended mean respiration rate (black line) using the fast Fourier transform non-linear least squares algorithm.

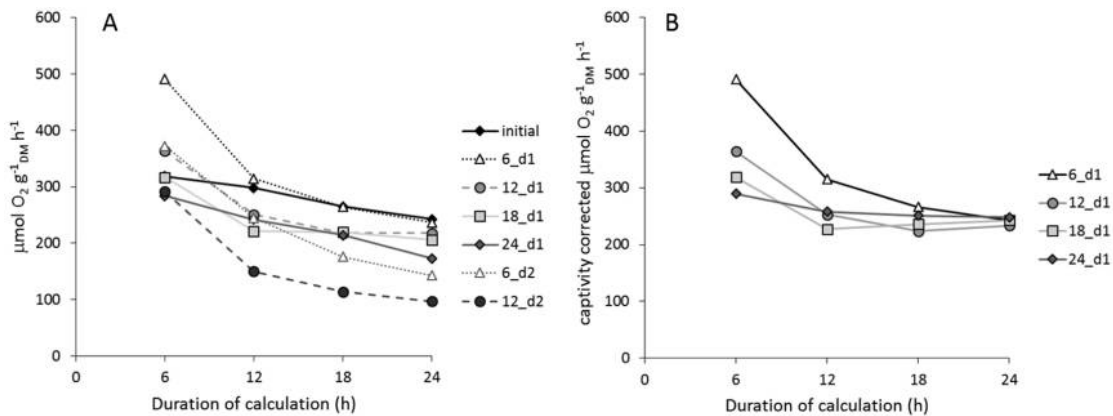


Figure 2. Effect of starting time and duration on rate calculation. The measured mass-specific metabolic rate (A) of *Pleuromamma xiphias* (y-axis) is plotted based on how long the experiment was run (x-axis) and using various starting points (the different lines correspond to the GMT time that the calculation was started). After correction for the decline in metabolic rate associated with captivity (B), it is obvious that the duration and time at which the experiment starts influence the calculated metabolic rate by up to 50%.

Using these corrected values and the average dry mass (DM) of the copepods (0.39 mg), we found a 24-h metabolic rate of $244 \mu\text{mol O}_2 \text{g}^{-1} \text{DM h}^{-1}$, with a daytime (06:00–18:00) rate of $315 \mu\text{mol O}_2 \text{g}^{-1} \text{DM h}^{-1}$ and a nighttime (18:00–06:00) rate of $220 \mu\text{mol O}_2 \text{g}^{-1} \text{DM h}^{-1}$. Animals ranged in size from 2.47 to 3.37 mg wet weight and from 0.34 to 0.42 mg dry weight.

Transcriptome

The assembly metrics for the transcriptome were comparable to other recent pelagic crustacean *de novo* transcriptomes and sufficient to allow for mapping of protein identification. After quality trimming, the assembly was generated from 267 million paired-end reads. Of these, less than 0.4% were shorter than the 25-bp cutoff that is Trinity’s default setting for input. The final assembly, in which highly similar sequences were clustered *via* CD-HIT (Li and Godzik, 2006) with a 95% similarity threshold, was composed of 554,991 Trinity transcripts and 458,857 Trinity “genes.” Of the original trimmed reads, 94.08% were aligned back to this reference assembly, with 72.89% aligning concordantly as proper pairs. This very large assembly consists of several transcripts expressed at very low levels, most of which are likely to represent noncoding sequences, rare variants, and assembly artifacts. Only 22.7% are expressed at a level of at least 1 transcript per million. The average length of the transcripts was 445 bp, with a median length 308 bp and an N50 of 469 bp but an E90N50 of 980. BUSCO analysis of the assembly reported a score of 89.3% completeness, with 16.2% duplication, 9.0% fragmentation, and 1.7% missing of 978 genes.

Annotation of the transcriptome with BLASTX *versus* nr (cutoff $1e^{-6}$) and Swiss-Prot (cutoff $1e^{-10}$), resulted in 22% and 16% annotation, respectively. Comparisons across other crustacean species using BLASTX at an e-value cutoff $1e^{-5}$ cross annotated anywhere between 14.5% (*Euphausia*

superba; Meyer *et al.*, 2015) and 30.1% (*Calanus finmarchicus*; Tarrant *et al.*, 2014) (Supplemental Data 2A, available online). The directed search for gene families of interest found matches for all previously identified genes that play roles in the major metabolic pathways, circadian rhythms, molting, and stress response for Crustacea (Christie *et al.*, 2013; Meyer *et al.*, 2015; Supplemental Data 2B, available online).

Proteome

Across all copepods analyzed, 1653 proteins were identified with high confidence in the proteomics data set (Supplemental Data 3, available online). Technical replicates showed a high degree of similarity, so NSAF values were averaged across replicates for the final analysis. Although there was no significant difference in full proteome profile between time points (ANOSIM, $R = 0.193$, $P = 0.076$; Fig. 3), there was a high similarity of protein abundance profiles for 3 individuals from the 20:00 time point, with 86% similarity compared to an average of 74% similarity among individuals (Fig. 3).

Of the 1653 proteins, 180 (~11%) were differentially abundant between the 2 time points with time-point-specific proteomic signatures. Ninety-seven of these proteins were detected at a higher abundance in copepods collected at 02:00 (positive log-fold change), and 83 were higher in copepods collected at 20:00 (negative log-fold change). Distinct classes of proteins were represented in these differentially abundant sets (Supplemental Data 3, available online). At 20:00 *P. xiphias* was enriched for biological processes involving gas and/or oxygen transport and molecular function that GO terms “oxygen transporter activity,” “iron ion binding,” and “helicase activity.” Proteins that were at significantly higher abundance in the 02:00 collected copepods were enriched for cellular components that included ribosome complexes and the molecular function “structural constituent of ribosome.”

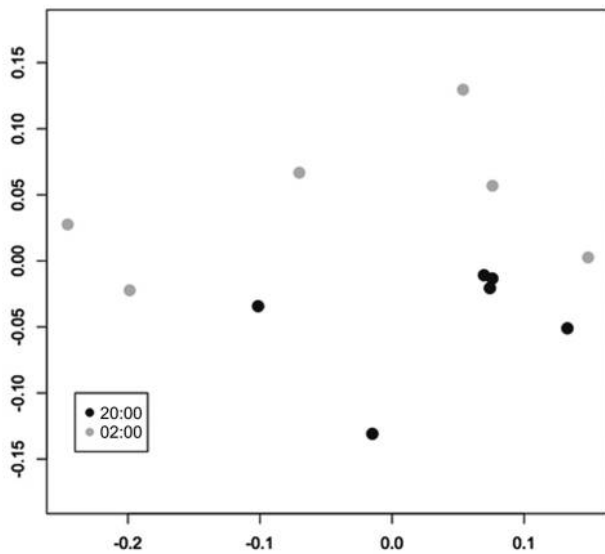


Figure 3. Non-metric multidimensional scaling (NMDS) plot of the abundance of complete proteome profile. Protein abundance values were $\log(x + 1)$ transformed, and a Bray-Curtis dissimilarity matrix was constructed to compare individuals. An NMDS plot was built from this matrix, where each point corresponds to one adult female *Pleuromamma xiphias*. 2D stress = 0.08.

At the level of individual proteins, there were several shifts in protein abundance that differentiated copepods from the two time points. Although differentially abundant proteins involved in transcription and translation and the cytoskeleton were present in both groups of copepods, they were much more prevalent in the surface-feeding animals at 02:00. DNA replication-related differentially abundant proteins were more prevalent in the upward migrators at 20:00 (7 vs. 2 differentially abundant proteins at 02:00). At each time point there were protein groups at higher abundance unique to that time point. At 20:00 the unique higher-abundance proteins were involved in the processes of lipid transport (2 proteins), bioluminescence (8 proteins), oxygen transport (4 proteins), shell matrix (1 protein), and hemolymph coagulation (1 protein). At 02:00 these were proteins involved in the muscle (2 proteins), lipid metabolism (4 proteins), nicotinamide adenine dinucleotide/reduced nicotinamide adenine dinucleotide (NAD/NADH) metabolism (4 proteins), and signal transduction (2 proteins). At both time points, proteins in a number of functional categories such as tricarboxylic acid (TCA) cycle proteins, adenosine triphosphate (ATP) binding and synthesis, carbohydrate metabolism, membrane proteins, calcium ion binding, reactive oxygen species response, and protein folding exhibited significant differential abundance.

Discussion

This study establishes a *de novo* transcriptome and proteome for one of the best-studied vertical migrators in the open ocean, *Pleuromamma xiphias*. These tools reveal changes in

physiology between early evening, when the copepods first reach the surface (20:00), and early morning, when the copepods have been actively feeding in surface waters (02:00). While equipment constraints precluded sampling wild-caught organisms from the daytime portion of the diel cycle, our proteomic and transcriptomic resources indicate the feasibility and value of a larger-scale experiment that samples the organisms from their daytime deep-water environment. Respiration experiments suggest a true circadian pattern in metabolism, emphasizing the utility of this organism for exploring the interaction between migration and circadian rhythms in diel vertical migratory plankton and indicating that future molecular analyses should include mid-morning and mid-night sampling to better constrain this circadian signal.

Respiration experiment

Despite the continuous decline in respiration rate, which is likely a consequence of captivity and food deprivation, there was a strong and statistically significant cyclical pattern in oxygen consumption for the four respiration trials. The pattern, which showed peak metabolism in the early to mid-morning and a nadir at mid-night, persisted in the absence of a light cue, suggesting that it is a true circadian rhythm. Without a light signal to provide entrainment, circadian patterns can become attenuated over time. This is particularly evident in peripheral tissues studied in culture (reviewed in Hastings *et al.*, 2003) but has also been described at the organismal level in diverse species, including copepods (Peres *et al.*, 2014; Maas *et al.*, 2016; Häfker *et al.*, 2017).

This variation in metabolism has important implications for our current calculations of respiratory carbon demand, for zooplankton active flux *via* diel migration, and possibly for calculations of zooplankton contributions to nitrogen supply in the midwater. Biogeochemical calculations (Dam *et al.*, 1995; Steinberg *et al.*, 2000, 2002; Schnetzer and Steinberg, 2002b) typically do not document the time during which bottle experiments were started, and experiments often integrate metabolic rates over 6–24-h periods. For example, Teuber *et al.* (2013) reported an average respiration rate of 50 and 116 $\mu\text{mol O}_2 \text{ g}^{-1}_{\text{DM}} \text{ h}^{-1}$ for adult (male and female, respectively) *P. xiphias* individuals from Cape Verde. These experiments were run 5–70 h after collection for a duration of 6–21 h and were performed with much larger individuals (0.87–1.82-mg males and 0.19–0.77-mg females compared with our 0.34–0.42-mg mixed gender). Steinberg *et al.* (2000) collected adult individuals (sex and individual dry mass undocumented) from the Sargasso Sea at night and ran experiments ~8 h in duration, measuring a respiratory rate of 1.6 $\mu\text{g C mg}^{-1}_{\text{DM}} \text{ h}^{-1}$ at 24 °C. Assuming a respiratory quotient of 0.87 (Mayzaud *et al.*, 2005), this yields a respiration rate of 139 $\mu\text{mol O}_2 \text{ g}^{-1}_{\text{DM}} \text{ h}^{-1}$. The rates from both of these studies are less than half of the metabolic rate we measured for the captivity-corrected 12-h daytime (06:00–18:00) portion of our incuba-

tion ($315 \mu\text{mol O}_2 \text{ g}^{-1}_{\text{DM}} \text{ h}^{-1}$). The respiration rate reported by Pavlova (1994) was an order of magnitude greater than that in our study, as well as those of Teuber *et al.* (2013) and Steinberg *et al.* (2000). Pavlova's (1994) highest respiration rate was $2500 \mu\text{mol O}_2 \text{ g}^{-1}_{\text{DM}} \text{ h}^{-1}$, with a daytime respiration rate of $312 \mu\text{mol O}_2 \text{ g}^{-1}_{\text{DM}} \text{ h}^{-1}$ and a similar dry weight as in the present study (0.29–0.39 mg).

Based on the results of our study, it is likely that an underestimation of the morning peak in the metabolic rate of *P. xiphias* contributed to the lower metabolic rates reported by both Teuber *et al.* (2013) and Steinberg *et al.* (2000), although duration of the experiments and differences in organismal mass, food availability, and recent exposure to hypoxia (in the case of the Teuber *et al.* [2013] experiments) likely also played a role. The active flux of carbon to the deep ocean *via* migrator respiration only occurs during the daytime hours when organisms are at depth. Shorter respiration experiments starting in the early evening or those that extend a full 24 h would underestimate daytime respiration by ~6%–24%, based on our calculations. This is important because these reported respiratory rates are scaled to a community-level response and are used to estimate carbon flux based on an assumption that respiration is largely controlled by temperature, body mass, and oxygen availability (Ikeda, 2014; Kiko *et al.*, 2016). Migrators of the genus *Pleuromamma* make up, on average, ~10% of the nighttime biomass (<200- μm size fraction) at BATS, with the species *P. xiphias* making up ~50% of the *Pleuromamma* biomass (Steinberg *et al.*, 2000). Thus, our findings suggest that there is likely an appreciable underestimation of the calculation of active flux in the Sargasso Sea due to circadian variation in *P. xiphias* respiration rate. Should the circadian pattern in metabolism observed in our experiment be a broadscale pattern in plankton physiology, then our estimates of the biogeochemical contributions of DVM species to midwater processes during the daytime portion of their DVM would be substantially underestimated.

The consistency of the circadian pattern remains uncertain, however, because there now have been a number of variations reported in vertically migratory copepods. Recent work done with *Calanus finmarchicus* corroborates our findings that calanoid copepods have a true circadian pattern in metabolic rate (Häfker *et al.*, 2017). The authors show a peak at 20:00 (dusk) and a nadir at 08:00 (3 h after dawn; mid-morning). It is difficult, however, to determine whether the patterns between these studies are similar, because the authors of the *C. finmarchicus* study plotted an anomaly of the decrease in oxygen rather than the respiration rate. Our findings are also somewhat different from the circadian pattern of diel vertically migratory copepods described by Pavlova (1994), who, during a study in the Indian Ocean between December and May, reported peaks in respiration about 2 h before sunrise (03:00–05:00) and 4–6 h after sunset (22:00–24:00), with a nadir between 12:00 and 16:00. Pavlova (1994) also reports a similar pattern for *Calanus euxinus* collected in September 1992 in

the Black Sea, with peaks at near sunrise (06:00) and 4 h after sunset (22:00) and a nadir at 12:00 and 02:00. Although these studies are difficult to compare, there does seem to be a consistent trend of increased respiration near dawn and dusk.

This trend is consistent with the idea that dawn and/or dusk metabolism would be the highest, as a consequence of the energetics associated with the migratory event. It is clear that DVM behavior is stimulated by light cues (reviewed in Cohen and Forward, 2009), and there is some evidence that DVM behavior is entrained by the light cycle and is under circadian control (Häfker *et al.*, 2017). In our study, the presence of a peak only at dawn, with the dusk respiration being close to the lowest point of metabolic rate measured, adds complexity to this interpretation. Furthermore, the nighttime metabolic minimum observed in this study is inconsistent with current ideas of DVM physiology, which would predict higher evening metabolism. During the day, it is assumed that migratory organisms are in a quiescent phase, saving energy in the cold, dark depths, where food is scarce, while they are actively feeding and growing in the warm surface waters at night (McLaren, 1963; Loose and Dawidowicz, 1994). *Pleuromamma xiphias* is, however, thought to be one of the species of diel vertical migrators that actively feeds on particles at depth (Schnetzer and Steinberg, 2002a). Thus, an increased metabolic activity during the day at depth may be related to their particular ecology. Irrespective as to the cause, this nonconformity of circadian response across studies indicates that there is species-specific, regional, or seasonal variation. Understanding the species-specific and geographic patterns, along with their ecological drivers, will be important in refining our understanding of daytime respiration for calculations of biogeochemical flux.

Transcriptome

Although very little is known about copepod transcriptomes, much of the available information suggests that they can be very large. Recently sequenced calanoid copepod transcriptomes that use similar sequencing technology and assembly methods range in number of transcripts from ~50,000 to 240,000 (Supplemental Data 4, available online). Our assembly, which consists of more than 400,000 unique “genes,” indicates that *P. xiphias* has a highly complex transcriptome in comparison to other calanoids. It is, however, very likely that many of the sequences are a consequence of short-read sequence-based assembly and the Trinity algorithms. Using analyses of transcript expression level (Ex) and associated weighted mean for a given expression level (ExN50), it is clear that sequences with extremely low abundance (between E99 and E100), which are difficult to assemble into long and meaningful contigs, make up 37% of the assembly. Choosing an Ex of only 98% puts the number of transcripts at ~238,000, while choosing an Ex of 90% reduces the number to ~52,000, well within the range of other assemblies. Despite the high number of rare transcripts, we feel that it is important to include all data in the assembly

because the biological relevance of these contigs is impossible to determine. Importantly for the proteomics mapping, the assembly appears to be relatively complete, with our BUSCO score similar to that of the gene set of other more “model” invertebrates, including *Caenorhabditis elegans* and *Lottia gigantea* (Simão *et al.*, 2015; Waterhouse *et al.*, 2018), although there was a higher proportion of duplicated orthologs (16.2% vs. 11.4% and 13.1%, respectively). *Pleuromamma* belongs to the Augaptilioidea, the basal planktonic superfamily within the Calanoida (Blanco-Bercial *et al.*, 2011; Bradford-Grieve *et al.*, 2014). Most of the other available calanoid transcriptomes belong to Centropagoidea (*e.g.*, *Eurytemora*) or Megacalanoida (*e.g.*, *Calanus*), both with very different ecological characteristics and evolutionary histories (Thuesen *et al.*, 1998; Bradford-Grieve, 2002; Takenaka *et al.*, 2012). To our knowledge, there is no other freely available annotated transcriptome for the Augaptilioidea to date, despite their prominence in open ocean environments and in the carbon and nitrogen export to the mesopelagic. This transcriptome thus adds a significantly different resource to the calanoid copepod genomic data available to the community.

Based on our analyses of transcripts expressed at low levels, it is likely that only about 23% of the transcripts were substantially expressed. Overall annotation *via* the nr and UniProt databases was somewhat limited, with a success rate of ~20%. The increased annotation success of the transcriptome when blasted against another copepod transcriptome (36% vs. *C. finmarchicus*) provides reassurance that the likely coding regions are well assembled but reiterates that copepod transcriptomes are highly complex and require further research attention (Bron *et al.*, 2011). The annotation of the assembly, which includes a directed search for homologs of known genes involved in molting, metabolic processes, stress response, and circadian rhythm, will allow for further analyses of these important processes.

Proteome

To our knowledge, this is the first study to annotate and measure more than a thousand proteins in a copepod proteome from single individuals, thus capturing inter-individual variability and highlighting the important proteomic physiological processes that are at play during surface residence time in these diel vertical migrators. Although there was no significant proteome-wide difference between the two time points, the differential abundance of copepod proteins revealed marked physiological signatures that were suggestive of changing metabolic processes, respiration, and antioxidant enzyme activity. It is important to note that individuals for the proteome study were collected only on one day for each time point, creating pseudoreplication in the experimental design. Furthermore, organisms were only sampled during the top portion of their diel migration and from two different days. These limitations make it difficult to attribute changing protein abundance ex-

clusively to circadian cycling or migratory activity. The results do reveal significant changes in a subset of the protein classes that were hypothesized to be sensitive to these factors, and they validate the use of these tools to investigate diel changes in migrator physiology.

Most previous proteomic work with copepods has explored the stress responses of individuals based upon the proteomic responses of tens to hundreds of pooled individuals in laboratory experiments (Kimmel and Bradley, 2001; Zubrzycki *et al.*, 2012; Boulangé-Lecomte *et al.*, 2016), with only one other study taking a shotgun proteomics approach (Wang *et al.*, 2017). The interpretation of these data is limited in ecological scope when *in situ* proteomes are uncharacterized and when inter-individual variability is masked by pooling. In contrast, the only proteomic studies on *in situ* copepod proteomes to date applied mass spectrometry methods with limited resolution (matrix-assisted laser desorption/ionization time-of-flight, MALDI-TOF) and limited interpretation for the purpose of species identification, rather than protein inference and physiology (Riccardi *et al.*, 2012; Laakmann *et al.*, 2013). Here, the characterization of individual proteomes from *in situ* samples reveals biologically and environmentally relevant physiological and metabolic patterns of an ecologically important species.

Although previous copepod studies do not report overall proteomic profiles to allow for direct comparison, the lack of overall proteomic differentiation is not surprising, as these proteomes were analyzed from wild-caught individuals that may vary in feeding history, reproductive state, precise age, and other factors. The number of proteins identified (1653) and the proportion that were differentially abundant (~11% of the proteome) were similar to what has been seen before when using a shotgun proteomics approach to study stress responses in other marine species. Wang *et al.* (2017) describe ~3100 proteins, ~2% of which were differentially abundant in the proteome of the copepod *Tigriopus japonicus* exposed to acidification and/or mercury. In a study of oysters exposed to acidification and/or mechanical stress, Timmins-Shiffman *et al.* (2014) described 1616 proteins, ~15% of which were differentially abundant.

The proteomes of the copepods that had recently arrived at the surface after migrating up from the depths (20:00 time point) showed evidence of increased oxygen transport, reproductive effort, and bioluminescence. Later in the evening (02:00) there was a higher abundance of a number of metabolic proteins, consistent with the carbohydrate, lipid, and general oxidative metabolism. *A priori* we hypothesized that we should see changes attributable to migration and circadian rhythmicity as variations in metabolic pathways, respiratory activity, antioxidant enzymes, and circadian proteins; and, thus, we focus the discussion on these patterns. With the exception of circadian proteins, there were significantly differentially abundant proteins associated with all of these functions in our analysis. Although sequences for circadian regulatory proteins were present in the transcriptome, most of these genes were expressed at

low levels (Supplementary Data 4, available online), and none of them were detected within the proteome. Studies conducted in model animal systems demonstrate that expression of core regulatory transcripts, such as *Clock* and *Period*, are highest in neural “pacemaker” tissues, and relatively low levels are needed to regulate the expression of their downstream targets. Due to the high sensitivity of transcriptional profiling methods, this approach may provide better characterization of circadian physiology in future studies.

The increased abundance of four proteins involved in oxygen transport and iron binding at 20:00 may reflect the increased demand for internal oxygen transport to meet ATP demand during the upward migration of copepods through the water column. Three of these proteins were uncharacterized, and one was annotated as a metalloprotein when blasted against the Swiss-Prot database; all four, however, showed homology to hemerythrin, an invertebrate oxygen-binding protein with two iron atoms (reviewed in Stenkamp, 1994), when blasted against the NCBI nr protein database on May 26, 2017. A comparable increase in a blood oxygen-binding protein to support seasonal migration has been shown in birds, where increased concentration of red blood cells (hematocrit) and increased hemoglobin have been documented for migratory species and individuals preparing for or undergoing migration (Piersma *et al.*, 1996; Landys-Ciannelli *et al.*, 2002; Minias *et al.*, 2013). Concurrently, 4 redox-associated proteins, including a protein disulfide-isomerase and a ubiquitin-activating enzyme, were detected at higher levels at 20:00, suggesting a signal of production of reactive oxygen species by these migrating individuals and the targeting of damaged proteins for degradation. Two other redox enzymes (dihydrolipoyl dehydrogenase and peroxiredoxin-6) were at increased abundance at the later 02:00 time point. Dihydrolipoyl dehydrogenase can have an antioxidant role by reducing ubiquinone to ubiquinol (Xia *et al.*, 2001). As such, it may be a second wave of redox response, returning the organism to a reduced state after the stress of migration. Peroxiredoxins, in contrast, are well-known antioxidant enzymes that undergo circadian cycles in redox state that are deeply conserved across the tree of life and are sometimes accompanied by circadian cycles in the protein abundance (Edgar *et al.*, 2012). Thus, although peroxiredoxin expression may be associated with an oxidative stress response, the variation in this protein may also be indicative of the natural circadian pattern of *P. xiphias*, and it is a good candidate for further exploration.

In the copepods that had been residing at the surface (collected at 02:00), there was proteomic evidence for increased digestive metabolism, where food is most abundant, with a higher abundance of carbohydrate metabolism proteins ($n = 7$ proteins) and lipid metabolism proteins ($n = 4$ proteins). One of these proteins, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, has previously been shown to have a diel cycle in dinoflagellates, with the enzyme abundance peaking in the late night and early day (Fagan *et al.*,

1999). More broadly, it is well established that there are circadian controls on many classes of genes associated with lipid metabolism and glucose homeostasis (Schlierf and Dorow, 1973; Marquez *et al.*, 2004; Rudic *et al.*, 2004). Thus, beyond changes associated with increased food availability in surface waters, this may be evidence of a circadian pattern in protein expression associated with metabolism in the late-evening copepods. Further analyses, controlling for the feeding state of the copepods and sampling over the diel cycle, will be required to determine whether light levels entrain these metabolic patterns independent of food availability.

Several proteins coding for enzymes directly involved in oxidative metabolism (NADH dehydrogenase, ATP synthase) were also at elevated levels at 02:00. They may similarly be linked to the circadian infrastructure associated with feeding. It has been demonstrated in mice that the circadian clock influences mitochondrial oxidative capacity *via* regulation of oxidized NAD (NAD⁺) biosynthesis and that these cofactors influenced lipid oxidation (Peek *et al.*, 2013). The increase in these proteins may also be a precursor to the early-morning peak in metabolism documented in the respiration experiment.

Conclusions

Classical studies of migration detail pronounced changes in organismal physiology associated with long-duration, long-distance migrations in a suite of terrestrial and marine systems. In contrast, organisms that travel short distances, or those that consume food as they travel, often do not show pronounced physiological specialization associated with migration (Blem and Gauthreaux, 1980). Zooplankton migrations, which occur over a short period of time but over a very large distance, provide a distinctly different class of migration that has not been physiologically well studied to date. Furthermore, due to their reliance on daily light cues, these migrations are linked to circadian signaling. Although brief in duration, they are thought to require the expenditure of a large amount of energy in a short period of time, and it is unclear whether specific changes in physiology occur before, during, and/or after the migratory events. Disentangling the physiology of migration and circadian rhythm from the pronounced changes in the vertical environment, which involve transitions from the warm, well-oxygenated, food-replete surface to the cold, dark, food-poor midwater, has previously been difficult because of a lack of adequate molecular tools for assessment and less sensitive oxygen sensors.

This study, which provides a *de novo* transcriptome that acts as a template for proteomics analysis, is a useful first step toward approaching these questions. Our results suggest several pathways (*e.g.*, respiratory oxygen-binding pigments, antioxidants, lipid, carbohydrate, and oxidative metabolism) that may respond to the migration event or be influenced by circadian signaling. With these tools in place, more substantial efforts, involving sampling over the full diel cycle at both surface and depth and/or sampling of cultured organisms under more

controlled environmental conditions, will help to resolve underlying patterns of true circadian rhythmicity and the specific physiological changes that occur as these organisms make their daily migration. Understanding these circadian and migratory patterns is important, because, as shown by our respiration data, they can have an important and overlooked influence on our calculations of biogeochemical fluxes.

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Data Accessibility

Sequence and assembly data associated with this project are archived in GenBank under BioProject PRJNA352670. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GFCI00000000. The version described in this paper is the first version, GFCI01000000.1. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD005778.

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